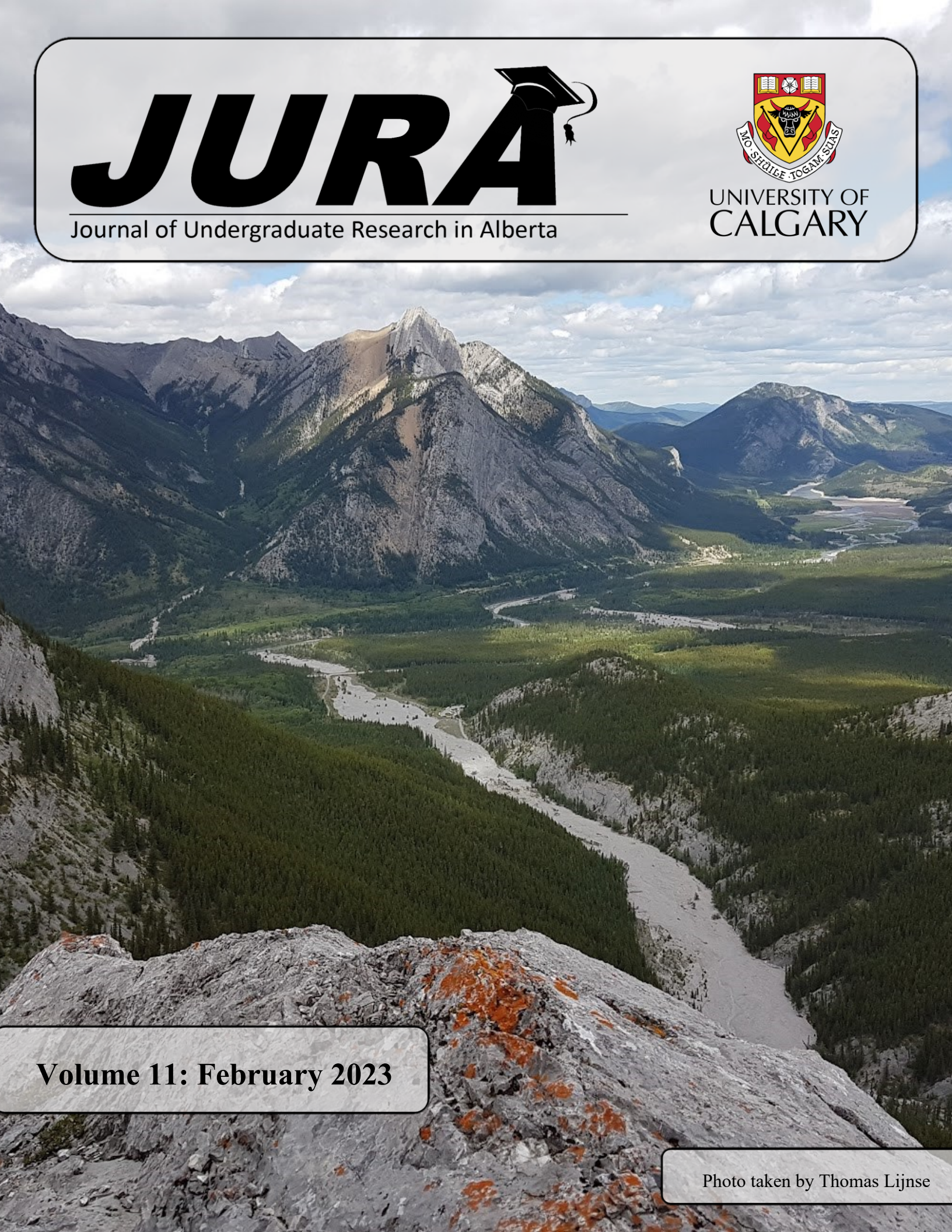
JURA

Journal of Undergraduate Research in Alberta

UNIVERSITY OF CALGARY



Volume 11: February 2023

Photo taken by Thomas Lijnse

Table of Contents

Volume 11: February 2023

Editors' Remarks

We are pleased to present the February 2023 full edition of the Journal of Undergraduate Research in Alberta. We continue to see and recognize the excellence of the undergraduate students that has proven vital to the advancement of our journal and we thank them for their contributions.

This edition features a articles from undergraduate researchers as well as the future of our scientific fields with articles from students in high school working in research labs. We at JURA are currently encouraging further submissions of research from undergraduate students within the science realm. Students are encouraged to submit written portions from sources beyond extracurricular laboratory research such as their thesis dissertations and class research projects. Furthermore, students are encouraged to continue submitting review papers, as these are equally vital contributions. Moving forward, JURA will aim to return to a regular publishing schedule with two issues per year in the summer and winter.

Regrettably, as our trainees progress we must inevitably leave the management of organizations such as JURA. We currently have only two members of our editorial board, one of whom will be leaving in order to defend. We are actively seeking to recruit new members for our editorial team, lest we be forced to go into a temporary hiatus. We look to continue serving the undergraduate research community, so we encourage any graduate students interested in getting their hands on an aspect of the peer review process to reach out.

Sincerely,

Jura Editorial Team

Thomas Lijnse

Sarah Kazemi

Investigating sarcomere length non-uniformity and force in immunofluorescent labelled sarcomeres from skeletal rabbit psoas muscles

Dhairya Desai[1,2], Armaan Sekhon[1], Timothy Leonard[1], Walter Herzog[1]

1 Human Performance Lab, University of Calgary, Calgary, AB, T2N 1N4, Canada
2 dhairya.desai@ucalgary.ca, 587-889-3400

Received 14 Oct 2022, revised 4 Jan 2023, accepted 4 Jan 2023, published 24 February 2023.

Abstract— Measuring sarcomere lengths (SLs) in skeletal muscle fibers and myofibrils has traditionally been done using bright-field and phase contrast (PC) microscopy. This often leads to inaccuracy and unrepeatability of measured SLs. Promising and reliable epitope labelling can be done on sarcomeric structural proteins α-actinin (Z-line) and myomesin (M-line). However, antibody labelling has been associated with reductions in myofibril function and active force production. Impacts of antibody labelling on sarcomere dynamics remain vague. The purpose of this study was to develop and test antibody labelling techniques and determine effects on sarcomere function, and development of SL non-uniformities. Myofibrils from the psoas muscle of New Zealand White rabbits were extracted. Labelled trials were incubated with anti-α-actinin, anti-myomesin primary antibodies, and polyclonal IgG (H+L) AlexaFluor488 secondary antibodies, then observed under an Olympus IX83 microscope using PC and Fluorescein isothiocyanate (FITC)-filtered microscopy. Myofibrils (Labelled, n=7 and Control, n=7) were attached to force-measuring cantilevers and stretched passively from a mean SL range of 2.5-2.6 µm (short position; SP) to 3.1-3.2 µm (long position; LP). Pearson correlation calculations on labelled (n=150) and control (n=141) sarcomeres between SLs yielded values of 0.80 (labelled), 0.48 (control) between SP1 and SP3, and 0.71 (labelled), 0.76 (control) for LP1 and LP3. Reliability of SL measurements for immunofluorescent labelled myofibrils was better at all positions (SP1, SP3, LP1, and LP3) compared to control sarcomeres. Force differences between labelled (n=6 myofibrils) and control (n=7 myofibrils) trials were not statistically significant (p=0.480). However, results suggest that introduction of antibodies may have caused increased friction in sarcomeres, observed by the decrease in SL non-uniformities at shorter lengths where passive stress produced is negligible and unable to overcome the increased friction. Antibody labelling did not impact sarcomere force production following passive stretch-shortening cycles. In conclusion, antibody labeling improved the precision and repeatability in SL measurements by clarifying sarcomere boundaries. Future investigation of antibody labelling in calcium-activated myofibrils is required to connect these findings to the active components of myofibrils.

I. INTRODUCTION

Muscle fibers contain rod-like organelles, called myofibrils, which host the functional units of contraction, the sarcomeres, connected mechanically in series within isolated myofibrils [1]. Sarcomeres are made up of many structural and contractile proteins that help them to achieve contraction. Sarcomere dynamics have been explored at the myofibril level and it has been observed that measuring methods tend to be slow, hard to reproduce, and have been associated with unrealistic accuracies [discussed in [2]]. Improved accuracy in sarcomere length (SL) measurements may help resolve some of the mechanical properties of muscles associated with SL instability and non-uniformity, such as residual force enhancement, which is the phenomenon where a muscle produces more force during an isometric contraction following active muscle stretching, compared to a purely isometric contraction at the same length and activation [3]. To potentially improve SL measurements, antibody labelling of sarcomeric structures has been explored. To be useful, antibodies labelling of sarcomeric structures should not interfere with the contracting elements of the sarcomere, thus force production should not be affected. Telley et al., 2006 [14] demonstrated that accurate epitope labelling can be made on the α-actinin and myomesin structural proteins, which span the Z-line and M-line of sarcomeres, respectively. Figure 1 provides a sarcomere diagram showing the contractile (actin, myosin, titin) and structural proteins (myomesin, α-actinin), as well as the Z-line, M-line, I-band, and A-band.

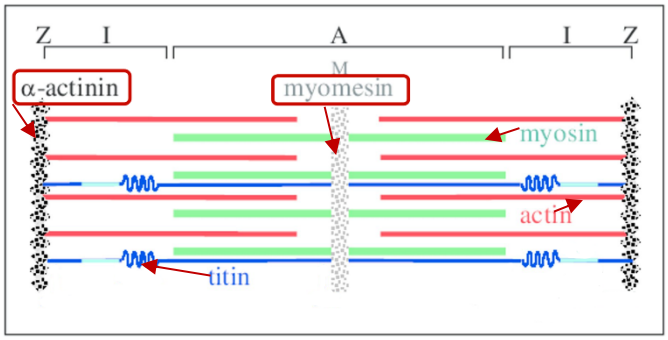


Fig. 1. Sarcomere structure with the major structural (α-actinin, myomesin) contractile proteins (actin, myosin, titin), and Z-line, I-band, A-band, M-line labelled [16].

SL non-uniformities in mammalian skeletal muscles occur on all structural levels: single myofibrils [5, 6, 20], single fibers [26] and entire muscles [27, 28, 29]. They are clearly present and are not a result of measurement errors or abnormalities in the muscle. SL non-uniformities have been associated to exist due to the differences in contractile and titin filament ratios between sarcomeres in a myofibril. Aside from the residual force enhancement property mentioned earlier, these non-uniformities have been associated with the creep behavior of muscles [30], residual force depression [31], SL instabilities [32], increased isometric force [25], and changes in the shape of the force-length relationship [12].

It is important to study the development of SL non-uniformities in muscles as it has been a phenomenon consistently observed throughout myofibril research. For half a century, residual force enhancement was exclusively associated with the development of SL non-uniformities when an active muscle was stretched on the descending limb of the force-length relationship [34,35]. Residual force depression is defined as the loss of isometric force following active muscle shortening compared to the isometric force of a purely isometric contraction at the same length and activation [10,34,36,37]. The sarcomere length non-uniformity theory (SLNT) has also been used to explain this property as differences in SLs are the reason behind varying force in the myofibril [21]. Recent research [6] has however shown that this may not be the case, where SLNT and instabilities exist in all muscle contractions but do not actually correlate with the residual force enhancement seen in these muscles. Nevertheless, it is still beneficial to confirm these results using the antibody labelling protocol, to increase reliability and accuracy of these findings, and to confirm if any of the other muscle-related properties can be supported by the SLNT.

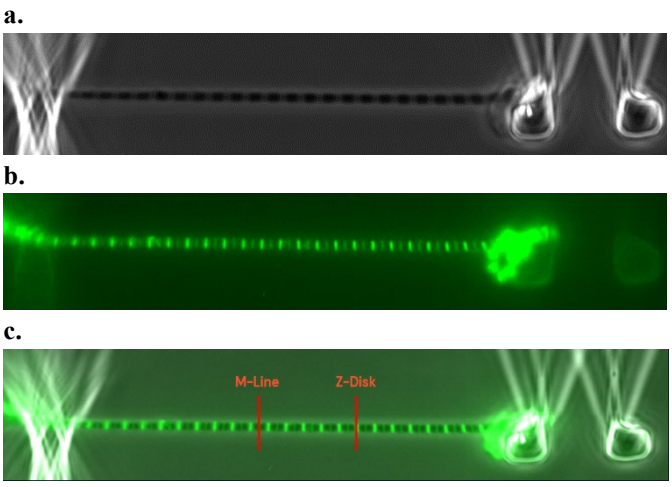


Fig. 2. Comparison between **a.** traditional bright-field and PC microscopy and **b.** antibody labelled FITC-filtered microscopy, with an overall **c.** overlap image of both methods. Note that Z-disk is equivalent to Z-line.

SL measurements have been done traditionally using bright-field and phase contrast (PC) light microscopy, measuring the distance between adjacent A-band or Z-line centroids [6]. These measurements rely on the accurate delineation of the Z-lines and borders of the A-bands (Figure 1) which results in variability of the SLs, as image quality is not always optimal, illumination varies across the myofibril, intensity profiles of Z-lines and A-bands drift, and the borders of Z-lines and A-bands are typically defined by constant but arbitrary thresholds [2]. Antibody labelling of the structural proteins at the M-Line and Z-line should provide more accurate and reliable measures of individual SLs by illuminating sarcomeric boundaries. Figure 2 provides an example of the effect of immunofluorescence microscopy compared to traditional bright-field and PC microscopy for the same myofibril held between force-measuring cantilevers and a glass needle. The difference between the two methods is clearly demonstrated in Figure 2, showing increased difficulty in accurately finding the Z-line in the bright-field and PC microscopy in each sarcomere.

Previous research [14,33] has yielded promising results in the specificity of antibody labelling. However, the impact of antibodies on passive and active myofibril forces and SLs non-uniformities have not been conclusive. Researchers identified either negligible reductions or two-fold reductions in active force per cross-sectional area. Turnover kinetics were similarly inconsistent. It is important to ensure that the addition of antibodies does not impair sarcomere dynamics, allowing the method to be a reliable SL measurement technique. The purpose of this study was to design and evaluate antibody labelling protocols primarily by determining measurement precision and explore the impact on dynamic sarcomere characteristics, such as the development of SL non-uniformities in passively stretched myofibrils.

II. Methods and Materials

A. Experimental Design

Small samples of tissue from eight New Zealand White rabbit psoas muscles were dissected, isolated, fastened to wooden sticks, and stored in a rigor solution (6 hours at 4°C) and then transferred to a rigor-glycerol solution, for 2 weeks at -20°C [18]. This method of storage does not affect results significantly. On the day of experiments, muscles were washed with and stored in rigor solution. Samples were cut from the fastened muscle and fractionated while suspended in rigor solution to purify them from connective tissues and extraneous organelles and expose single myofibrils. These treated samples were incubated with anti-α-actinin (A7811, Sigma-Aldrich) and anti-myomesin (mMaC myomesin B4, Developmental Studies Hybridoma Bank) primary antibodies, then incubated with polyclonal IgG (H+L) AlexaFluor488 antibodies (A32723, Fisher Scientific). Samples were then observed under an inverted Olympus IX83 microscope (Olympus) using phase-contrast imaging and FITC-filtered microscopy on a jacuzzi stage that can add and drain relaxing solution. A microscopic glass needle and force-measuring cantilevers (with silicon-based adhesive) were used to hold the myofibril in place for stretches to take place. Data was collected under 100x magnification with a resolution of 65 nm/pixel.

B. Solutions

Rigor solution [5, 18]: Tris (50 mM), NaCl (100 mM), KCl (2 mM), MgCl2 (2 mM), and ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 10 mM) at pH 7.0.

Relaxing solution [5, 18]: Imidazole (10mM), MgCl2 6H2O (4 mM), disodium creatine phosphate (Na2CrP; 47.7 mM), dithiothreitol (DTT; 2 mM), EGTA (3 mM), KCl (6 mM), and sodium adenosine triphosphate (Na2ATP; 1 mM) at pH 7.0.

Activating solution [5, 18]: Imidazole (10mM), MgCl2 6H2O (4 mM), disodium creatine phosphate (Na2CrP; 47.7 mM), dithiothreitol (DTT; 2 mM), EGTA (3 mM), CaCl2 (3 mM), and sodium adenosine triphosphate (Na2ATP; 1 mM) at pH 7.0.

C. Ethics

New Zealand White rabbits were euthanized by an intravenous injection of 1 ml of sodium pentobarbital (240 mg/mL) according to a protocol approved by the Life and Environmental Sciences Animal Care Committee of the University of Calgary (Ethics ID: AC19-0155), and the psoas muscle was collected for study purposes.

D. Passive Stretch Experiments

Myofibrils were flushed with relaxing solution and suitable myofibrils (distinct striation patterns with I-bands, A-bands, and Z-lines clear) were picked up using a glass needle and attached to a cantilever using a silicon-based adhesive to capture images and timelapses. Passive stretches were performed where the myofibril was suspended and held at an average SL (obtained by measuring the myofibril length and dividing it by the number of sarcomeres) range of 2.5-2.6 μm (short position – SP) and then stretched and held at an average SL range of 3.1-3.2 μm (long position – LP). These specific lengths were chosen because at SP, there should be no passive force, whereas at LP, there should be measurable passive force. At both lengths, the stretch was performed slowly and held for 10 seconds, to ensure stabilization of the cantilever for accurate force measurements. The entire stretch relaxation experiment was recorded in timelapse (intervals of approximately 0.250 s) and performed 3-4 times per myofibril. The timelapse feature on the Olympus IX83 was used to capture and overlay the phase-contrast imaging with the FITC-filtered microscopy.

E. Sarcomere Length Non-Uniformity

SL measurements were taken using a built-in ruler in the CellSens Dimensions software for the Olympus IX83 calibrated using accurate pixel-to-μm ratios. The maximum number of sarcomeres accurately measurable in each myofibril were chosen, and sarcomeres were pooled together from the seven control and seven antibody-labelled myofibrils, resulting in n=141 control sarcomeres and n=150 antibody-labelled sarcomeres. For passive stretches, timelapses were collected by stretching the myofibril from SP, and then repeatedly stretching to LP. Individual SL measurements were measured from adjacent M-line centroids throughout the myofibril at SP and LP. SL non-uniformity was measured by measuring SLs after repeated passive stretches and observing if sarcomeres would return to their original lengths, in both control and antibody-labelled myofibrils.

F. Myofibril Force Measurements

Force was determined using a microfabricated pair of cantilevers, as proposed by Fauver et al., 1998 [15], where one end of the myofibril was attached using a mixture of silicon-based adhesive to one of the cantilevers, while the other end of the myofibril was pierced using a glass needle. As passive stretches occurred, the relative movement of the cantilever relative to the stationary cantilever was measured [15] and force calculated based on the known stiffness of the cantilevers. The cross-sectional area of the myofibril was determined using the average measured diameter along the myofibril, assuming a cylindrical cross section, and with the myofibril held at an average SL of 2.7 μm [18]. The cross-sectional area was used to determine the longitudinal stress in the myofibril ($nN/\mu m^2$).

G. Repeatability

Reproducibility of the SL measurements was determined by repeated analysis of the same sarcomeres three times using 7 labelled myofibrils (n=150 sarcomeres, resulting in a total of n=450 measurements) and 7 control myofibrils (n=141 sarcomeres, resulting a total of 423 measurements). SLs between repeated trials were compared to themselves. The three measurements were made at two-week intervals, and the standard deviation between SL measurements for these three repeat measurements were taken as the outcome of reproducibility of our SL measurements. The standard deviation between control and antibody-labelled repeated SL measurements was compared to assess the difference in reliability of SL measurements with the novel antibody-labelling method.

A. Statistical Analysis

Please note that each myofibril has 20-30 sarcomeres in series and isolating a single sarcomere from a myofibril to do experiments would be close to impossible due to the experimental apparatus. When doing experiments, sarcomeres in series were measured and analyzed. The SLNT is tested for the sarcomeres within a myofibril, as it is the difference in SLs within an individual myofibril, and this trend is found in all levels of muscle.

Myofibril Force:

Passive stress was calculated using force and cross-sectional area of myofibrils from control (n=7 myofibrils), measured three times over two weeks (n=21 measurements) and labelled (n=6 myofibrils) measured three times over two weeks (n=18 measurements), and a paired samples t-test was performed to quantify significance between labelled and control stress values. Measurements were tested three times repeatedly to ensure result reliability. Force was calculated by measuring displacement between cantilever pair following passive stretches, and the known stiffness constant of the cantilever. Cross-sectional area was calculated by measuring the diameter of the myofibril.

B. Sarcomere length non-uniformity:

SLs were followed through repeated passive stretches. observing if they return to original length and form original SL non-uniformities in the myofibrils. Linear regression curves and Pearson correlation (R^2) calculations were performed to demonstrate if SLs return to their original lengths after passive stretch disruptions. An identity line showing ideal correlation is used to compare findings. Experimental myofibrils were compared to the control reference.

Repeatability:

SL measurements of the same 7 control and 7 labelled myofibrils (from eight rabbits) were taken three times over 2-week intervals and mean standard deviations between repeated measurements of the same sarcomere were compared to measure reliability of SL measurements. Experimental myofibrils were compared to the control reference.

III. Results

A. Repeatability

Measurements of SLs were more accurate and reliable (smaller mean standard deviation between measurement repeats) in the labelled than the control myofibrils for all conditions (Figure 3). The mean standard deviation of the repeated measurements is statistically less in antibody-labelled sarcomeres compared to controls shown in Figure 3. The bars represent the mean standard deviation between SL measurements repeated 3 times at SP1, SP3, LP1, LP3 for control (n=423 measurements) and antibody-labelled (n=450 measurements) sarcomeres. The error bars represent standard error of the mean standard deviation, and overlap was used to assess statistical significance.

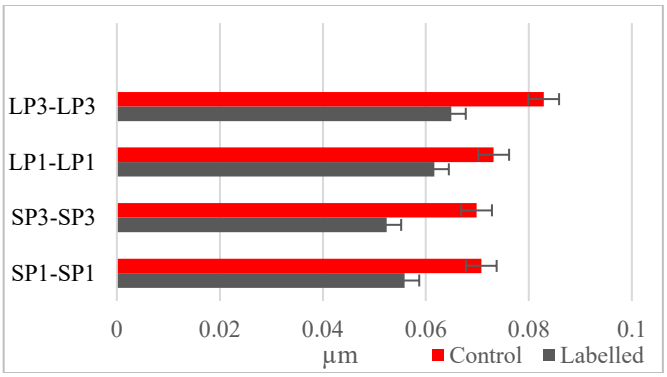


Fig. 3. Bars represent mean standard deviation of repeated same SL measurements (µm) (n=3) in the short positions (SP) and the long positions (LP) (µm), in antibody labelled (n=7) and unlabelled control (n=7) myofibrils, over a 6-week period. Standard error bars used to assess significance.

B. Force Production

Myofibril passive force production had no significant differences between labelled and control myofibrils (Fig. 5), and paired t-test resulted in a *p*-value of 0.480.

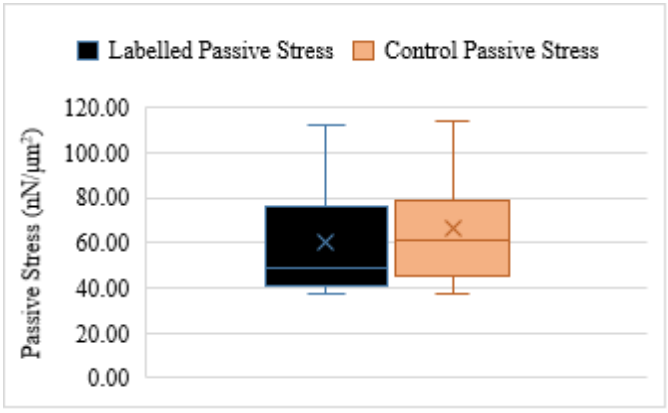


Fig. 5. Boxplots comparing passive stress of control and antibody-labelled myofibrils in long positions (average SL range of 3.10-3.24 µm).

C. Sarcomere Length Non-Uniformity

Myofibril SLs began at, and returned to, initial lengths following passive stretch disruptions, presented by the linear regression curves in Fig. 4. R^2 values were calculated using the preset statistics software in Microsoft Excel. Similar R^2 values were observed in sarcomeres in control myofibrils at long (mean SL range of 3.1-3.2 µm) and short (mean SL range of 2.5-2.6 µm) positions, and in sarcomeres in the labelled myofibrils at LP with the R^2 being 0.76, 0.71, 0.80, respectively. This was not followed in sarcomeres at shorter lengths in labelled myofibrils with the R^2 being 0.48, and the data points further spread from the identity line.

IV. Discussion

A. Repeatability

The reduced standard deviations of repeat SL measurements in the labelled compared to the control myofibrils suggests that antibody labelling of the Z-lines and M-bands indeed improves detection of these structures, resulting in better reliability of SL measurements. This further validates the usage of the antibody-labelling method in sarcomere analysis, as similar SLs were obtained through repeated analysis, proving consistency and accuracy of this method of sarcomere analysis. By using antibodies to label proteins spanning the centroids of sarcomeres, it is possible to obtain increased measurement accuracy, when coupled with immunofluorescence microscopy. This helps to support the use of antibody-labelling in future myofibril research to account for the repeatability aspect of experiments.

D. Force Production

There was no statistical difference (p=0.480) in passive force between control and labelled myofibrils, suggesting that the introduction of antibodies did not impact passive force production, in limited passive stresses.

E. Sarcomere Length Non-Uniformity

The SLs at LPs for both control and labelled myofibrils, as well at SPs in the labelled myofibrils were comparable with similar R^2 correlations. Their graphs showed results close to the identity line, which helps to demonstrate that the SLs were returning to their initial positions. This indicates that the original SL non-uniformity within the myofibril is being maintained following passive stretch disruptions. However, the SLs at SPs for the labelled myofibrils had a significantly smaller R^2 value, and trends in the graph where the points were increasingly clustered at the mean SL and did not return to the identity line. This signifies a decrease in the original SL non-uniformity where sarcomeres are not returning to the length they started at before the passive stretch disruptions.

This observation can be potentially attributed to an increase in friction caused by the addition of antibodies to the sarcomere. As the correlation was weaker in relaxed positions where sarcomeres are already in a compacted state than in stretched positions where sarcomeres are elongated, the addition of antibodies may be interfering with the lattice structure of the sarcomere, causing the observed impairment to SL restoration dynamics.

Looking at the minute scale of a working sarcomere is important to further speculate reasoning behind the observed finding. An average sarcomere is about 1.5 to 3.5 µm [24], with several contractile and structural proteins. The proteins being labeled are a-actinin, which is about 35 nm long [23] and myomesin, about 50 nm long and 4 nm wide [23]. An antibody has an average size of 10 nm [22], and since both a primary and a secondary antibody was used in this protocol, each antibody addition required about 20 nm of space within the M-line and the Z-line. The saturation of the sarcomere with antibodies allows for a maximum number of antibodies to attach to their respective receptors within the sarcomere. This may be causing an increased frictional force within the sarcomere. However, the impact from this force was not seen at longer SLs because some of the passive force produced by the myofibril may be negating the increased friction, whereas at shorter lengths, there is essentially no passive force being produced by the myofibril. The sarcomere would therefore resist the return to original shorter lengths.

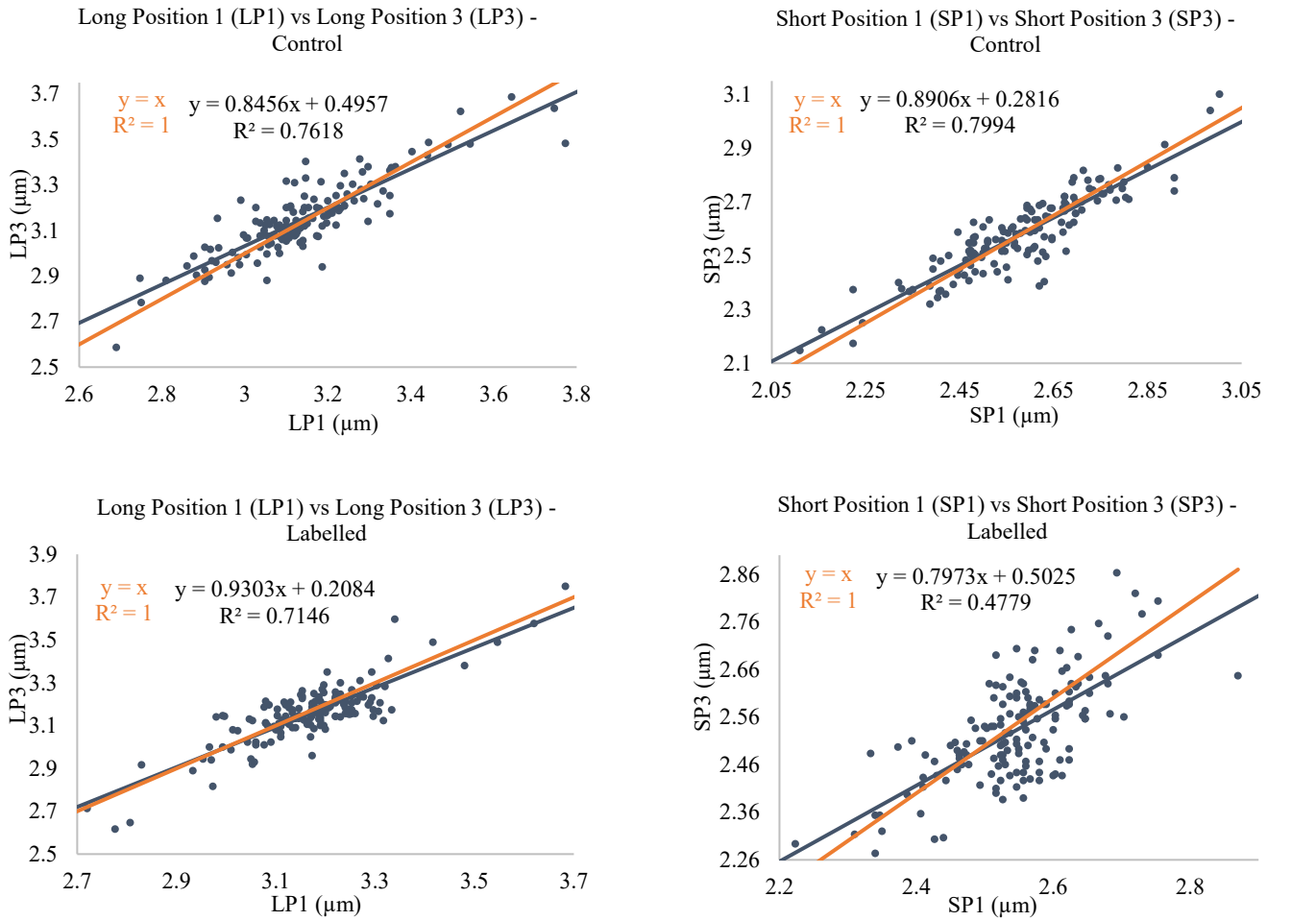


Fig. 4. SLs at an initial position compared to a similar position (short or long) following repeated passive stretches in labelled (n=7 myofibrils, n=150 sarcomeres) and control trials (n=7 myofibrils, n=141 sarcomeres). The orange line was an identity line used as a theoretical SL reference to demonstrate complete correlation compared to experimental SL results

V. Conclusions

B. Summary of Findings

Overall, the introduction of antibodies did not constrain sarcomere dynamics in the passive force produced by the myofibril following passive stretches. It did, however, cause a decrease in the development of SL non-uniformities at shorter lengths. The antibody labelling method increased the precision of SL measurements, indicated by the decreased standard deviation between repeated measurements for the labelled myofibrils. These findings help support further use of this method in myofibril research.

C. Study Significance and Limitations

Previous research using antibodies has been performed [14,17]. However, the results in these studies differ, especially in regard to changes in force caused by the introduction of antibodies in a myofibril. Future research should test for the effects of antibody labeling on active force. If the introduction of antibodies does not impact the sarcomere dynamics, it would represent a powerful tool to study molecular details of muscle contraction mechanisms.

Fluorescent antibodies are subject to photobleaching, presenting a limitation on the data that can be collected from antibody labelled samples. Adjusting antibody concentrations may be necessary to increase the signal strength without impacting sarcomere functionality. It may also be beneficial to try to use signalling primary antibodies instead of secondary antibodies, as this would reduce the number of proteins added to the myofibril, and still provide the same effect of antibody labelling as the method used in this study protocol.

The antibody labelling method used in this study provides a consistent visual increase in precision for myofibril research and should be used to increase repeatability of SL measurements in myofibril experiments. Further experiments are required to a) explore the effects of antibody labeling in activated myofibrils, and b) find ways to mitigate the observed decrease in SLNT at shorter lengths.

D. Future Directions

These results suggest that further investigation in calcium-activated experiments is warranted to explore the effects in active muscle experiments. However, overall, the a-actinin and myomesin labelling method used in this study does serve as a confirmation to explore potential antibody labelling methods for contractile proteins within the sarcomere, such as labelling different segments of titin, actin, or myosin.

ACKNOWLEDGMENT

I would like to express my thanks to Armaan Sekhon, Dr. Walter Herzog, Dr. Tim Leonard, for the contributions to experiments, consultation for ideas, and revisions to this paper, as well as Dr. Venus Joumaa and the rest of the Human Performance Lab for providing support and guidance throughout the summer. I would also like to thank the funding I received from NSERC's Undergraduate Student Research Award that made this research possible.

REFERENCES

[1] W. R. Frontera and J. Ochala, "Skeletal Muscle: A Brief Review of Structure and Function," Calcified Tissue International, vol. 96, no. 3, pp. 183–195, Oct. 2014, doi: 10.1007/s00223-014-9915-y.

[2] J. Schmidt, A. Jinha, and W. Herzog, "Sarcomere length measurement reliability in single myofibrils," Journal of Biomechanics, vol. 126, p. 110628, Sep. 2021, doi: 10.1016/j.jbiomech.2021.110628.

[3] W. Herzog, G. Schappacher, M. DuVall, T. R. Leonard, and J. A. Herzog, "Residual Force Enhancement Following Eccentric Contractions: A New Mechanism Involving Titin," Physiology, vol. 31, no. 4, pp. 300–312, Jul. 2016, doi: 10.1152/physiol.00049.2014.

[4] T. Iwazumi and G. H. Pollack, "The effect of sarcomere non-uniformity on the sarcomere length-tension relationship of skinned fibers," Journal of Cellular Physiology, vol. 106, no. 3, pp. 321–337, Mar. 1981, doi: 10.1002/jcp.1041060302.

[5] K. Johnston, A. Jinha, and W. Herzog, "The role of sarcomere length non-uniformities in residual force enhancement of skeletal muscle myofibrils," Royal Society Open Science, vol. 3, no. 3, p. 150657, Mar. 2016, doi: 10.1098/rsos.150657.

[6] K. Johnston, E. K. Moo, A. Jinha, and W. Herzog, "On sarcomere length stability during isometric and post-active-stretch isometric contractions," Journal of Experimental Biology, vol. 222, no. 22, Jan. 2019, doi: 10.1242/jeb.209924.

[7] J. W. Krueger and G. H. Pollack, "Myocardial sarcomere dynamics during isometric contraction.," The Journal of Physiology, vol. 251, no. 3, pp. 627–643, Oct. 1975, doi: 10.1113/jphysiol.1975.sp011112.

[8] E. K. Moo and W. Herzog, "Sarcomere Lengths Become More Uniform Over Time in Intact Muscle-Tendon Unit During Isometric Contractions," Frontiers in Physiology, vol. 11, May 2020, doi: 10.3389/fphys.2020.00448.

[9] E. K. Moo, T. R. Leonard, and W. Herzog, "In Vivo Sarcomere Lengths Become More Non-uniform upon Activation in Intact Whole Muscle," Frontiers in Physiology, vol. 8, Dec. 2017, doi: 10.3389/fphys.2017.01015.

[10] R. A. M. Pinnell, P. Mashouri, N. Mazara, E. Weersink, S. H. M. Brown, and G. A. Power, "Residual force enhancement and force depression in human single muscle fibres," Journal of Biomechanics, vol. 91, pp. 164–169, Jun. 2019, doi: 10.1016/j.jbiomech.2019.05.025.

[11] J. G. Pinto and P. J. Patitucci, "Creep in cardiac muscle," American Journal of Physiology-Heart and Circulatory Physiology, vol. 232, no. 6, pp. H553–H563, Jun. 1977, doi: 10.1152/ajpheart.1977.232.6.h553.

[12] W. Herzog, E. J. Lee, and D. E. Rassier, "Residual force enhancement in skeletal muscle," The Journal of Physiology, vol. 574, no. 3, pp. 635–642, Jul. 2006, doi: 10.1113/jphysiol.2006.107748.

[13] J. A. Talbot and D. L. Morgan, "Quantitative analysis of sarcomere non-uniformities in active muscle following a stretch," Journal of Muscle Research and Cell Motility, vol. 17, no. 2, pp. 261–268, Apr. 1996, doi: 10.1007/bf00124247.

[14] I. A. Telley, J. Denoth, E. Stüssi, G. Pfitzer, and R. Stehle, "Half-Sarcomere Dynamics in Myofibrils during Activation and Relaxation Studied by Tracking Fluorescent Markers," Biophysical Journal, vol. 90, no. 2, pp. 514–530, Jan. 2006, doi: 10.1529/biophysj.105.070334.

[15] M. E. Fauver, D. L. Dunaway, D. H. Lilienfeld, H. G. Craighead, and G. H. Pollack, "Microfabricated cantilevers for measurement of subcellular and molecular forces," IEEE Transactions on Biomedical Engineering, vol. 45, no. 7, pp. 891–898, Jul. 1998, doi: 10.1109/10.686797.

[16] V. Person, S. Kostin, K. Suzuki, S. Labeit, and J. Schaper, "Antisense oligonucleotide experiments elucidate the essential role of titin in sarcomerogenesis in adult rat cardiomyocytes in long-term culture," Journal of Cell Science, vol. 113, no. 21, pp. 3851–3859, Nov. 2000, doi: 10.1242/jcs.113.21.3851.

[17] M. DuVall, "Titin Regulation of Active and Passive Force in Skeletal Muscle," prism.ucalgary.ca, Sep. 2015, doi: 10.11575/PRISM/28459.

[18] V. Joumaa, F. Bertrand, S. Liu, S. Poscente, and W. Herzog, "Does partial titin degradation affect sarcomere length nonuniformities and force in active and passive myofibrils?," American Journal of Physiology-Cell Physiology, vol. 315, no. 3, pp. C310–C318, Sep. 2018, doi: 10.1152/ajpcell.00183.2017.

[19] D. Morgan, "An explanation for residual increased tension in striated muscle after stretch during contraction," Experimental Physiology, vol. 79, no. 5, pp. 831–838, Sep. 1994, doi: 10.1113/expphysiol.1994.sp003811.

[20] D. E. Rassier, W. Herzog, and G. H. Pollack, "Dynamics of individual sarcomeres during and after stretch in activated single myofibrils," Proceedings of the Royal Society of London. Series B: Biological Sciences, vol. 270, no. 1525, pp. 1735–1740, Aug. 2003, doi: 10.1098/rspb.2003.2418.

[21] R. Haeger, F. de Souza Leite, and D. E. Rassier, "Sarcomere length non-uniformities dictate force production along the descending limb of the force–length relation," Proceedings of the Royal Society B: Biological Sciences, vol. 287, no. 1937, p. 20202133, Oct. 2020, doi: 10.1098/rspb.2020.2133.

[22] M. Reth, "Matching cellular dimensions with molecular sizes," Nature Immunology, vol. 14, no. 8, pp. 765–767, Jul. 2013, doi: 10.1038/ni.2621.

[23] R. Craig and R. Padrón, "Chapter 7 Molecular Structure of the Sarcomere S100A4-Myosin IIA Dynamics View project Myosin 3D reconstruction View project," 2014.

[24] R. M. Enoka and J. Duchateau, "Muscle Function: Strength, Speed, and Fatigability," Muscle and Exercise Physiology, pp. 129–157, Jan. 2019, doi: 10.1016/B978-0-12-814593-7.00007-4.

[25] J. C. K. Wells and M. S. Fewtrell, "Measuring body composition," Archives of Disease in Childhood, vol. 91, no. 7, pp. 612–617, Jun. 2005, doi: 10.1136/adc.2005.085522.

[26] A. F. Huxley and L. D. Peachey, "The maximum length for contraction in vertebrate striated muscle," The Journal of Physiology, vol. 156, no. 1, pp. 150–165, Apr. 1961, doi: 10.1113/jphysiol.1961.sp006665.

[27] M. E. Llewellyn, R. P. J. Barretto, S. L. Delp, and M. J. Schnitzer, "Minimally invasive high-speed imaging of sarcomere contractile dynamics in mice and humans," Nature, vol. 454, no. 7205, pp. 784–788, Jul. 2008, doi: 10.1038/nature07104.[28]E.

[28] K. Moo, R. Fortuna, S. C. Sibole, Z. Abusara, and W. Herzog, "In vivo Sarcomere Lengths and Sarcomere Elongations Are Not Uniform across an Intact Muscle," Frontiers in Physiology, vol. 7, p. 187, 2016, doi: 10.3389/fphys.2016.00187.

[29] E. K. Moo and W. Herzog, "Single sarcomere contraction dynamics in a whole muscle," Scientific Reports, vol. 8, no. 1, Oct. 2018, doi: 10.1038/s41598-018-33658-7.

[30] A. M. Gordon, A. F. Huxley, and F. J. Julian, "The variation in isometric tension with sarcomere length in vertebrate muscle fibres," The Journal of Physiology, vol. 184, no. 1, pp. 170–192, May 1966, doi: 10.1113/jphysiol.1966.sp007909.

[31] D. L. Morgan, N. P. Whitehead, A. K. Wise, J. E. Gregory, and U. Proske, "Tension changes in the cat soleus muscle following slow stretch or shortening of the contracting muscle," The Journal of Physiology, vol. 522, no. 3, pp. 503–513, Feb. 2000, doi: 10.1111/j.1469-7793.2000.t01-2-00503.x.

[32] A. V. Hill, "The mechanics of active muscle," Proceedings of the Royal Society of London. Series B, Biological Sciences, vol. 141, no. 902, pp. 104–117, Mar. 1953, doi: 10.1098/rspb.1953.0027.

[33] Y. Shimamoto, M. Suzuki, S. V. Mikhailenko, K. Yasuda, and S. Ishiwata, "Inter-sarcomere coordination in muscle revealed through individual sarcomere response to quick stretch," Proceedings of the National Academy of Sciences, vol. 106, no. 29, pp. 11954–11959, Jul. 2009, doi: 10.1073/pnas.0813288106.

[34] B. C. Abbott and X. M. Aubert, "The force exerted by active striated muscle during and after change of length," The Journal of Physiology, vol. 117, no. 1, pp. 77–86, May 1952, Accessed: Oct. 15, 2022. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1392571/

[35] K. A. Edman, G. Elzinga, and M. I. Noble, "Residual force enhancement after stretch of contracting frog single muscle fibers.," The Journal of General Physiology, vol. 80, no. 5, pp. 769–784, Nov. 1982, doi: 10.1085/jgp.80.5.769.

[36] G. Maréchal and L. Plaghki, "The deficit of the isometric tetanic tension redeveloped after a release of frog muscle at a constant velocity.," The Journal of General Physiology, vol. 73, no. 4, pp. 453–467, Apr. 1979, doi: 10.1085/jgp.73.4.453.

[37] W. Herzog and T. R. Leonard, "Depression of cat soleus forces following isokinetic shortening," Journal of Biomechanics, vol. 30, no. 9, pp. 865–872, Sep. 1997, doi: 10.1016/s0021-9290(97)00046-8.

Comparison of COVID-19's Impact on Persons with Dementia Experiencing Responsive Behaviours During the First and Second/Third Waves of the Pandemic

Rhea Bhalla[1], Gwen McGhan[2], Kristin Flemons[3], and Deirdre McCaughey[3]

1 Notre Dame High School, Calgary, AB, T3K 6E4, Canada

2 Faculty of Nursing, University of Calgary, Calgary, AB, T2N 4N1, Canada

3 Faculty of Medicine, University of Calgary, Calgary, AB, T2N 4N1, Canada

Received Sept 22 2022, revised 8 Dec 2023, accepted 8 Dec 2023, published 24 February 2023.

Abstract— *Background:* The COVID-19 pandemic has placed immense pressure on people living with dementia people living with dementiaand the family care partners who support them. Public health measures, though vital to managing viral spread, may have a profound impact on the frequency of responsive behaviours experienced by people living with dementia, further amplifying negative effects of the pandemic. Responsive behaviours are the actions, words, and gestures used by people living with dementia as they respond to changes in their environment. The aim of this study was to use a comparative mixed methods research design to better understand the effects of the pandemic and related public health measures on responsive behaviours experienced by people living with dementia during the first and second/third waves of the COVID-19 pandemic in Alberta, Canada. Data was reported by family care partners for people living with dementia. ***Methods:*** Data collection was completed through an initial first wave pilot survey (n = 217) and focus groups conducted from June to September 2020, and a broader second/third wave provincial survey (n = 283) and focus groups conducted from February to May 2021. Participants were family care partners of people living with dementia during the first and/or second/third waves of the pandemic. Both qualitative and quantitative methods were utilized for data analysis. ***Results:*** Compared with the first wave, family care partners during the second/third wave of the pandemic described greater increases in the responsive behaviours experienced by the people living with dementia they cared for, with the second/third wave sample reporting increases in eleven of the twelve responsive behaviour categories. The primary themes that emerged in the qualitative analysis were isolation and loneliness (experienced by people living with dementia and family care partners), feelings of depression (experienced by people living with dementia and family care partners), progression of dementia, andlack of care and stimulation (experienced by people living with dementia). ***Conclusions:*** Since the onset of the COVID-19 pandemic, people living with dementia have faced significant increases in the frequency of responsive behaviours they experience, resulting in greater care provision challenges for their family care partners.

I. INTRODUCTION

Dementia, a clinical syndrome characterized by deterioration in cognitive function, is a major cause of dependency, disability, and death among older adults[1]. In 2015, 47 million people worldwide suffered from dementia, and that figure is projected to increase to 135 million by 2050[2]. The high prevalence of dementia across the globe and lack of disease-modifying treatments have contributed to a growing health concern[3]. As Canada's population ages and the number of people living with dementia rises exponentially[4], the increased demands on society, individuals, and healthcare systems present an ongoing challenge. Family care partners, who are relied upon to provide ongoing support for people living with dementia, will be especially affected.

In December 2019, the first cases of an unknown pneumonia emerged in China's Hubei province. The virus has since been identified as severe acute respiratory syndrome coronavirus 2 (COVID-19) pneumonia and has forced many countries to implement public health measures to mitigate the pathogen's aggressive spread and fatal consequences[5]. These measures include quarantines, lockdowns, social distancing, prohibition of travel, and the usage of personal protective equipment to reduce viral transmission rates. Older adults and vulnerable segments of the population have been profoundly impacted. According to Statistics Canada, dementia or Alzheimer's disease was reported on 36% of COVID-19 death certificates in 2020, making it the most common co-morbidity associated with COVID-19-related fatalities. This trend may be driven largely by age, since 94% of Canadians who died of COVID-19 in 2020 were over the age of 65[6]. Not only are people living with dementia generally more vulnerable to COVID-19, but they also have limited access to accurate information about the pandemic and may face difficulties in remembering safety procedures such as wearing masks and maintaining personal hygiene[7]. When unable to understand and follow public health measures, a person living with dementia could be exposed to a higher risk of infection. Though public health measures play a pivotal role in ensuring the safety of people living with dementia, family care partners' reduced access to caregiving resources, such as homecare and community programs, may lead to increased difficulty in fulfilling their role. In some cases, family care partners may not be able to provide care due to restrictions that limit access to people living with dementia.

An assessment of changes in health outcomes of people living with dementia is needed to gain a fuller understanding of the pandemic's effect on people living with dementia and the family care partners who support them. Responsive behaviours, those behaviours experienced by people living with dementia as they communicate their needs and respond to their personal, social, or physical environment[8], are an indicator of these outcomes. Responsive behaviours can involve delusions, hallucinations, apathy/indifference, or resisting care, and can cause distress to both people living with dementia and family care partner(s)[9]. Family care partners provide continuous hands-on care and support and are crucial to maintaining a person living with dementia's quality of life[10]. Caregiving is a strenuous role, with family care partners for older adults being at increased risk for burden,

stress, depression, and a variety of other health complications[11].

This research has been completed with the use of raw data collected by a larger parent study*, Conducting a Gap Analysis of Family Caregivers' Needs During a Global Pandemic*. The parent study, conducted by McGhan et al. from the University of Calgary's Faculty of Nursing, assesses the effect of the pandemic on family care partners for people living with dementia across the caregiving continuum. The caregiving continuum refers to a person living with dementia's place of residence (at home, in an assisted living facility, or in a long-term care facility). People living with dementia in long-term care facilities were especially likely to experience distress during the pandemic, due to the loss of family visits and limitations on participation in social events and recreational activities[12]. This research aims to explore the impact of COVID-19 on people living with dementia experiencing responsive behaviours, through analysis and comparison of quantitative and qualitative data gathered during the first and second/third waves of the pandemic. Results from the present study will allow for a better understanding of the unique challenges faced by people living with dementia who have been severely affected by the pandemic, particularly those experiencing responsive behaviours.

II. METHODS

A. Study Design

The pilot study was conducted from June to September 2020, following the peak of the first wave of the COVID-19 pandemic in Canada[13]. Data were collected through an online survey containing both multiple choice and short-answer questions as well as focus groups conducted in a virtual setting. Surveys were distributed in Southern Albertawith the assistance of community partners, namely the Alzheimer Society of Calgary and the Dementia Network Calgary. A total of $n = 217$ family care partners participated in the first wave survey. The second provincial survey built upon the first wave study and was expanded to include family care partners across the province of Alberta, in order to examine regional factors that may impact pandemic experiences of people living with dementia (i.e. differences in urban, suburban, and rural communities). In collaboration with the Community Advisory Committee, modifications were made to the first wave survey to capture a more accurate representation of family care partners' experiences during later pandemic waves. The study was conducted from February to May 2021, during the second/third wave of the pandemic[14]. Similar to the first wave study, participants in the second/third wave study completed an online survey, during which they were asked about their interest in an optional focus group component. Focus group participants were drawn from those who consented and provided contact information. A total of $n = 283$ self-selected family care partners participated in the second/third wave survey. To better guide the parent study, a Community Advisory Committee that included the Alzheimer Society of Calgary, the Dementia Network Calgary, the Alzheimer Society of Alberta and Northwest Territories, and Caregivers Alberta was convened. Community-based research strategies were used to engage key stakeholders across Alberta in study planning, execution and dissemination of findings. Research ethics approval for the parent study was attained from the Conjoint Health Research Ethics Board at the University of Calgary (REB20-0855).

B. Participants

The inclusion criteria were people over the age of 18 who identified as family care partners for a person living with dementia. Proficiency in English was required to successfully read, interpret, and respond to the survey. Informed consent was obtained from all participants, with a separate consent form distributed to family care partners involved in focus groups. Participants were not directly compensated for their participation but were notified of the vital role their input played in helping researchers learn more about supporting family care partners for people living with dementia during the COVID-19 pandemic, which could allow the province to better plan for future public health crises.

III. RESEARCH

A. Quantitative Data Collection

When completing the online survey, family care partners were given a list of 12 responsive behaviours that a person living with dementia may experience and were asked to indicate how the frequency of that behaviour had changed during the pandemic. Questions were adapted from the World Health Organization's COVID-19 Snapshot Monitoring (COSMO) survey, which sought to examine perceptions of pandemic-related risk, trust in health authorities, adherence to public health measures, and knowledge of COVID-19[15]. If a behaviour had not been experienced before or during the pandemic, the option "not applicable" was given. The following responsive behaviours were assessed: delusions, hallucinations, agitation/aggression, depression/dysphoria, anxiety, elation/euphoria, apathy/indifference, disinhibition, irritability/lability, motor disturbance, irregular nighttime activity, and change in appetite/eating behaviours. Responses were measured on a 3-point Likert scale[16], with the option to indicated that the person living with dementia's responsive behaviour "decreased," "remained the same," or "increased." Two additional responses, "I don't know" and "not applicable" were not included in statistical analysis of responsive behaviour changes. Family care partners were also asked to indicate whether their feelings of strain, feelings of isolation, and quality of life changed during the pandemic, stating whether these aspects were "better than usual," "about the same as usual," or "worse than usual." In order to examine relationships in the data during the quantitative analysis stage, participants were asked questions regarding the demographic groups they identify with. Four demographics were examined in this analysis: (1) age of person living with dementia, (2) gender of person living with dementia, (3) presence or absence of financial hardships caused by the COVID-19 pandemic, and (4) person living with dementia's position on the care continuum.

B. Quantitative Data Analysis

Data from the first and second/third wave studies were analysed in separate datasets using IBM SPSS Statistics for Windows,

version 28 (IBM Corp., Armonk, N.Y., USA). Frequencies were obtained using descriptive statistics to gain a comprehensive overview of the data and determine numerical values for the participant demographics. Values given by the descriptive statistics were then compared across the first and second/third wave datasets to explore differences in the first and second/third wave samples. Within a dataset, independent sample t-tests were used to assess the range in overall change in the responsive behaviours experienced by people living with dementia across a certain demographic category. Tested variables were inputted as one responsive behaviour score, and grouping variables were inputted as a specific demographic category. Each category was assessed separately in an individual independent variable t-test. For demographic categories involving more than two subsections, a one-way ANOVA (Analysis of Variance) was conducted. Information gained through the ANOVA included sum of squares, mean square, Levene's statistic based on mean, trimmed mean, and median with and without adjusted degrees of freedom. All statistical analysis were conducted with a confidence interval percentage of 95%. A significance (Sig.) value less than 0.05 on Levene's Test for Equality of Variances was considered statistically significant.

C. *Qualitative Data Collection*

Focus groups were conducted to gain greater insight into the experiences of family care partners. Sessions ranged from 60 to 90 minutes in length. Following the focus group session, audio recordings were de-identified and professionally transcribed verbatim for analysis.

D. *Qualitative Data Analysis*

Using the audio transcripts from the first and second/third wave focus groups, data were qualitatively analysed using a thematic approach. Thematic analysis is a method of understanding qualitative data that entails identifying, interpreting, and describing repeated patterns in a dataset[17]. A data-driven, inductive approach was employed to draw themes from participants' responses and minimize experimenter bias[18]. Coding was done by two members of the research team (R.B. and K.F.) in accordance with the six-step process outlined by Braun and Clarke (2006): (1) become familiar with the data, (2) generate initial codes, (3) search for themes, (4) review themes, (5) define themes, (6) complete write-up. A second coder reviewed all coded transcripts and any discordance on the meaning of the codes or themes was resolved through discussion to reach consensus on the coding structure. Prolonged exposure to the data and continuous discussion between the two coders allowed for a comprehensive analysis of emerging themes. These themes were further explored during larger team meetings and refined using insight provided by the Community Advisory Committee.

IV. DATA

A. *Quantitative Results*

Most family care partners who participated in the study identified the person living with dementia whom they cared for as being a woman. This was consistent across both the first and second/third wave studies, with 61.1% (n = 132) and 60.6% (n = 166) of people living with dementia being women, respectively. In the first wave study, over half of the people living with dementia were equal to or older than the age of 81 (54.6%, n = 118), slightly less than the provincial study, where that number was increased to 62.8% (n = 172). Financial hardships due to the COVID-19 pandemic were faced by 17.7% (n = 38) of participants in the first wave study, and 20.7% (n = 57) of participants in the provincial study. Table 1 displays sample characteristics of participants who completed the first wave survey and/or the second/third wave survey, and descriptive statistics related to the primary variables of interest.

Table 1. Sample Characteristics of Participants

		First Wave	Second/Third Wave	
Demographics	n	%	n	%
Age of Person Living With Dementia				
51-60 years	3	1.4	4	1.5
61-70 years	27	12.5	31	11.3
71-80 years	68	31.5	67	24.5
81+ years	118	54.6	172	62.8
Gender of Person Living With Dementia				
Man	84	38.9	106	38.7
Woman	132	61.1	166	60.6
Financial Hardships				
Yes	38	17.7	57	20.7
No	177	82.3	219	79.3

The frequency analysis showed 69.0% (n = 149) of the first wave sample reporting an increase in feelings of isolation during the pandemic, compared with 71.3% (n = 199) of the second/third wave sample. About two-thirds of the first wave sample (66.4%; n = 144) reported an increase in feelings of strain, slightly greater than the second/third wave sample (63.1%; n = 176). Quality of life was reported to have decreased by 55.3% (n = 119) of the first wave sample. For participants during the second/third wave, this number increased to over two-thirds, or 67.6% (n = 186) of the sample experiencing a decline in their quality of life during the pandemic.

Family care partners during the second/third wave of the pandemic described greater increases in the responsive behaviours experienced by the person living with dementia they cared for, with a larger portion of the second/third wave sample reporting increases in responsive behaviours such as delusions (55.4%; +16.9% from first wave), hallucinations (47.9%; +19.5% from first wave), agitation/aggression (55.2%; +11.4% from first wave), depression/dysphoria (61.6%; +18.4% from first wave), anxiety (61.8%; +9.3% from first wave), apathy/indifference (60.9%; +14.6% from first wave), irritability/lability (54.4%; +13.5% from first wave), disinhibition (32.1%; +5.2% from first wave), motor disturbance (50.6%; +18.0% from first wave), irregular nighttime activity (56.5%; +15.5% from first wave), and change in appetite/eating behaviours (25.0%; +13.3 from first wave). Only

one of the twelve responsive behaviour types, "elation/euphoria," saw fewer people living with dementia experience the behaviour during the second/third wave (5.0%) compared with the first (9.6%). This increase in responsive behaviours may be linked to a loss of caregiving resources used by family care partners to support people living with dementia. Data from the wave one study suggests that care partners went from using an average of five support resources before the pandemic to 1.6 during the pandemic. Changes in the frequency of responsive behaviours experienced by people living with dementia between the first and second/third wave are indicated in Fig. 1.

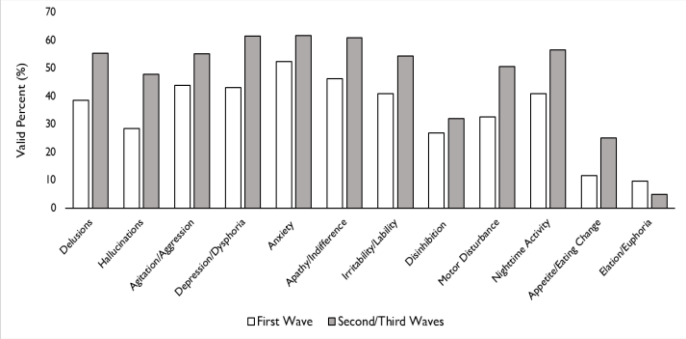


Fig. 1. People Living With Dementia Experiencing Increases in Responsive Behaviours

Between the first wave and second/third wave samples, four tested variables from the first wave survey responses presented with variances below 0.05 on the Levene's Test for Equality of Variances, and therefore presented with statistical significance. The one-way ANOVAs conducted for "position on the care continuum," "change in quality of life," and "family care partner isolation" revealed significance values (sig. based on mean) of <0.001. Though more heterogenous, the one-way ANOVA conducted for "family care partner strain" had a statistically significant value (sig. based on mean) of 0.019. All other demographics, including those tested for the second/third wave samples, presented with no statistical significance on the Levene's Test for Equality of Variances. Regardless of their place of residence, people living with dementia across the care continuum experienced increases in responsive behaviour frequencies and were negatively impacted by the pandemic.

B. Qualitative Results

Four main themes emerged from the qualitative analysis. The first theme, "isolation and loneliness," was present in responses discussing isolation, loneliness, or reduced opportunities for socialisation experienced by the person living with dementia as a result of the COVID-19 pandemic. The second theme, "progression of dementia," was present in responses discussing a deterioration of condition or increased adverse psychological and physical symptoms experienced by the person living with dementia. The third theme, "feelings of depression," was present in responses that identified feelings of depression, sadness, or lack of interest in activities once enjoyed by the person living with dementia. The fourth theme, "lack of care and stimulation," was present in responses discussing a person living with dementia's lack of access to care-related resources, activities, and other sources of enrichment. The themes, definitions, and illustrative quotes from the qualitative analysis are summarised in Table 2.

Table 2. Themes, Definitions, and Representative Quotes

Theme	Definition	Representative Quote
Isolation and Loneliness	Identifies responses that discuss isolation, loneliness, or reduced socialisation experienced by the person living with dementia as a result of the COVID-19 pandemic, especially for a prolonged period of time.	"The isolation broke her spirit."
Progression of Dementia	Identifies responses that discuss a deterioration of condition or increased adverse psychological and physical symptoms experienced by the person living with dementia.	"I would say [the COVID-19 pandemic] probably took six months off her life for sure, maybe more so."
Feelings of Depression	Identifies responses that discuss feelings of depression, sadness, or lack of interest in activities experienced by the person living with dementia, especially for a prolonged period of time.	"You could see her just sort of everything shutting down cause [sic] there's nothing."
Lack of Care and Stimulation	Identifies responses that discuss a lack of access to care-related resources, activities, and other sources of enrichment as a result of the COVID-19 pandemic.	"All the activities within the building under lockdown ceased . . . and that was a really hard thing for her to go through."

Theme 1, Isolation and Loneliness: Over two-thirds of family care partners during both the first and second/third waves of the COVID-19 pandemic reported an increase in feelings of isolation (69.0% and 71.3%, respectively). Not unexpectedly, a major theme that emerged in the focus groups was feelings of isolation, loneliness, and reduced socialisation experienced by people living with dementia because of the pandemic and related restrictions. For example, one family care partner during the first wave shared, "I think COVID meant that she [had] far less friends coming and less social stimulation for about a month and a half, and I think that created a bit of a downward spiral for her." Other care partners touched on their own feelings of isolation during the pandemic, with one participant stating, "It [the pandemic] was a very difficult, isolating time. And everybody felt that."

Theme 2, Progression of Dementia: Family care partners expressed concern regarding possible deterioration of condition or increased adverse psychological and physical symptoms experienced by the person living with dementia. Caregivers shared that the COVID-19 pandemic may have negatively impacted the person living with dementia's health and prognosis, causing decline

beyond what may have been expected in the absence of a global health emergency. One family care partner said, "We know that social interaction and stimulus is a contributing factor to progression... I'm really hard pressed to see how her decline isn't somewhat correlated to those kind of restrictions that ended up being in place... I also am worried that those restrictions have exacerbated her decline." Some care partners linked the decline in condition of the person living with dementia they care for to specific public health measures. For example, one family care partner said, "There has been a big decline in my mom's cognitive abilities over this last six months... Time is a factor, but I think it's also partially because her day programs are gone."

Theme 3, Feelings of Depression: During focus groups, some family care partners shared that the person living with dementia they care for experienced feelings of depression and/or sadness, especially for a prolonged period of time. For example, one family care partner said, "I could see her not wanting to live anymore." Family care partners also talked about concerns that people living with dementia no longer seemed interested in hobbies and activities they used to enjoy. One care partner said, "He didn't do anything. He wouldn't take part in programs or anything like that. And then when COVID came on that was a good excuse to just sit and do nothing, which is what he does. So he looks out the window and half the time I can't even talk to him." Another care partner said, "My wife lost interest [in the TV] over time in those last three months that we were locked because she just was having a hard time relating to the TV... She's not reading the newspaper and she's not getting anything out of the TV anymore because of her slide downhill."

Theme 4, Lack of Care and Stimulation: Family care partners reported frustration with the reduced access to care-related resources, activities, stimulation, and other sources of enrichment as a result of the COVID-19 pandemic. This may be due to a reduction in family care partners' access to support resources such as day programs, support groups, end of life planning, legal services, and transportation. One care partner said, "The biggest impact was not being able to go to appointments... I found that we delayed making appointments because it was either just virtual [or] by phone. And I felt that it needed [to be] more one-on-one personal... it didn't seem critical so we just have put off going. But the assessment process needs a one-on-one... kind of gathering. So that's been a real challenge because I see more slipping. And having that opportunity to go in has been a big challenge." Another family care partner stated, "My mom was lucky enough to end up in an excellent home... they allowed our care partner to stay on as an essential worker and so we had the comfort of knowing that there was a consistent person there through COVID for my mom... All of these poor, poor people weren't going outside anymore, weren't getting walked down the hallways, so people that were once upon a time walking, five months later are now in wheelchairs, or they've forgotten how to walk or they can't walk anymore."

V. DISCUSSION

In this research, data obtained from the parent study were analysed using a comparative mixed methods approach to present an in-depth exploration of people living with dementia experiencing responsive behaviours and the family care partners who have supported them during the first and second/third waves of the pandemic. Interview data from family care partners for people living with dementia and responses from two surveys were used to form a robust picture of how COVID-19 has affected this at-risk population.

Analysis of both the first and second/third wave samples found that people living with dementia during the COVID-19 pandemic experienced an increased frequency of responsive behaviours. Most notably, these increases were greater in eleven of the twelve second/third wave samples compared to the first wave, indicating that people living with dementia were more likely to experience worsening responsive behaviours in later waves of the pandemic. This may be a result of prolonged exposure to public health measures. Hindmarch et al.[19] emphasized the role of family care partners as key care partners for people living with dementia and suggested that care partners be supported with the necessary equipment and training while they continue to provide essential care. Despite the importance of stringent public health measures to minimize infection risks, it is equally important to address the reduction of care-related resources and supports that has been an unintended consequence of these restrictions, and take steps to prioritize the health and wellbeing of people living with dementia during the COVID-19 pandemic. Data from the wave one study showed that family care partners have experienced a marked loss of support resources, making it more difficult for them to provide people living with dementia with the care they need.

Family care partners in the second/third wave sample reported greater feelings of isolation and strain compared with those during the first wave. The qualitative results echo these findings, with care partners identifying sources of stress and concern that provide further context to quantitative data. Concerns over social isolation and reduced access to caregiving resources and supports were a major theme discussed during focus groups. Simonetti et al.[20] found that social restrictions and disruption of routine caused by the pandemic may lead to the onset or worsening of responsive behaviours that put people living with dementia at increased risk of self-injury, personal distress, COVID-19 transmission, and death. A 2020 report conducted by the Alzheimer Society of Ireland[21] emphasized the growing vulnerability faced by family care partners and people living with dementia arising from the cessation of caregiving resources and supports. Findings from the study highlight significant challenges for family care partners, such as cancellation or postponement of medical appointments, loss of routine, boredom and anxiety, and a marked increase in responsive behaviours experienced by the person living with dementia for whom they care. Prior to the onset of COVID-19, numerous studies have indicated strong correlations between dementia caregiving and negative effects on psychological health[22,23,24]. Now, more than ever, family care partners must be recognized and supported as key partners in care[19] for people living with dementia.

Examining the relationship between qualitative and quantitative data obtained from this study provides further context to the findings. The underlying theme of isolation and loneliness may contribute to a second theme of feelings of depression experienced by people living with dementia. Not only is loneliness recognized

to contribute to feelings of depression[25], it is a risk factor for all-cause dementia, especially Alzheimer's dementia[26]. Moreover, older adults who are lonely experience more severe depressive symptoms, compromised health, and a greater number of co-morbities[27]. Taken with the theme of people living with dementia lacking care and stimulation, these themes may help explain family care partner's concerns of dementia progression in the person living with dementia they care for. These concerns are mirrored by the results of the quantitative analysis, which identified an increase in responsive behaviours during the pandemic. While the quantitative data analysis provides insight into the impacts of the pandemic, information provided by family caregivers during focus group discussions offers explanations for these impacts and allows for the development of effective solutions.

Although the findings of this research may be interpreted as providing evidence for negative physiological and psychological effects of the pandemic, a true causal relation is not proven. Factors other than the pandemic (such as natural progression of dementia, growing older during the study, etc.) could have led to the higher rates of increasing responsive behaviours found in this study. It should be noted, however, that the results of the present study are roughly in line with systematic reviews of studies on the impact of the pandemic on people living with dementia[27]. Those studies report an increase in responsive behaviours (sometimes referenced as neuropsychiatric symptoms) experienced by people living with dementia appearing to arise from social restrictions occurring because of the pandemic. Therefore, it is likely that the increases in responsive behaviours and challenges faced by family care partners observed in the present study are related to the COVID-19 pandemic.

Considering the results of both the quantitative and qualitative analyses, it is clear that family care partners recognize the importance of social interaction to maintain the health and wellbeing of people living with dementia. To minimize the exacerbation of responsive behaviour frequencies, it is crucial that long-term care facilities ensure sufficient opportunities for socialization. This may be accomplished through providing family care partners with access to people living with dementia in a manner that prevents viral spread[28]. Likewise, it is important to reduce strain placed on family care partners by restoring dedicated services and facilities for people living with dementia as soon as possible, as many have been inactive or reduced in their activities during the pandemic[29].

VI. CONCLUSION

As the COVID-19 pandemic continues to challenge vulnerable older adults, it is important to understand how people living with dementia and their family care partners have been affected. By collecting data from family care partners during both the initial and later waves of the pandemic, this study established that people living with dementia have experienced greater responsive behaviour increases in the second/third wave of the pandemic, with major themes of isolation and loneliness, progression of dementia, feelings of depression, and lack of care and stimulation emerging as underlying factors. Furthermore, results indicate that family care partners have become continually more stretched in their caregiving roles, with the second/third wave sample reporting increased feelings of isolation, increased feelings of strain, and poorer quality of life during the pandemic. Collectively, the findings of this study suggest that COVID-19 has amplified the frequency of responsive behaviours and rate of symptom progression experienced by this sample of people living with dementia during the pandemic, and has placed immense burden on the people, systems, and support networks that care for them.

ACKNOWLEDGMENTS

Funding for the first wave parent study was received from the Alzheimer Society of Calgary. Funding for the second/third wave parent study was received from the Drummond Foundation.

REFERENCES

1. World Health Organization. (2017). Global action plan on the public health response to dementia, 2017-2025. https://www.who.int/publications/i/item/global-action-plan-on-the-public-health-response-to-dementia-2017---2025
2. Ricci G. (2019). Social Aspects of Dementia Prevention from a Worldwide to National Perspective: A Review on the International Situation and the Example of Italy. *Behavioural neurology*, *2019*, 8720904. https://doi.org/10.1155/2019/8720904
3. Prince, M. J., Wimo, A., Guerchet, M. M., Ali, G. C., Wu, Y. T., & Prina, M. (2015). World Alzheimer Report 2015-The Global Impact of Dementia: An analysis of prevalence, incidence, cost and trends.
4. Duong, S., Patel, T., & Chang, F. (2017). Dementia: What pharmacists need to know. *Canadian pharmacists journal : CPJ = Revue des pharmaciens du Canada : RPC*, *150*(2), 118–129. https://doi.org/10.1177/1715163517690745
5. Wang, C., Horby, P. W., Hayden, F. G., & Gao, G. F. (2020). A novel coronavirus outbreak of global health concern. *The lancet*, *395*(10223), 470-473.
6. Statistics Canada. (2021). Provisional death counts and excess mortality, January 2020 to February 2021. https://www150.statcan.gc.ca/n1/en/daily-quotidien/210514/dq210514c-eng.pdf?st=iyWbF5Lx
7. Wang, H., Li, T., Barbarino, P., Gauthier, S., Brodaty, H., Molinuevo, J. L., Xie, H., Sun, Y., Yu, E., Tang, Y., Weidner, W., & Yu, X. (2020). Dementia care during COVID-19. *Lancet (London, England)*, *395*(10231), 1190–1191. https://doi.org/10.1016/S0140-6736(20)30755-8
8. Brémault-Phillips, S., Friesen, S., Moulton, L., Germani, T., Miciak, M., Parmar, J., & Rogers, L. G. (2015). Managing responsive behaviours: a wicked problem addressed through a grassroots approach. *Healthc Q*, *18*(2), 31-5.
9. Buhr, G. T., & White, H. K. (2006). Difficult behaviors in long-term care patients with dementia. *Journal of the American Medical Directors Association*, *7*(3), 180–192. https://doi.org/10.1016/j.jamda.2005.12.003
10. Brodaty, H., & Donkin, M. (2009). Family caregiversof people with dementia. Dialogues in clinical neuroscience, 11(2), 217–228. https://doi.org/10.31887/DCNS.2009.11.2/hbrodaty.
11. Cassie, K.M., & Sanders S. (2008). Familial caregivers of older adults. In: Cummings SM, Kropf, Nancy P, eds. *Handbook of Psychosocial interventions with Older Adults: Evidence-Based Approaches*. Philadelphia, PA: Haworth Press; 2008:293–320.
12. Bianchetti, A., Rozzini, R., Bianchetti, L., Coccia, F., Guerini, F., & Trabucchi, M. (2022). Dementia Clinical Care in Relation to COVID-19. Current Treatment Options in Neurology, 1-15.
13. Foote, A., & Long, J. (2021, June 28). 15 months, 3 waves: How COVID-19 coursed through the capital. *CBC News*. https://www.cbc.ca/news/canada/ottawa/covid-coronavirus-pandemic-waves-ottawa-1.6075233

14. Detsky, A. S., & Bogoch, I. I. (2020). COVID-19 in Canada: experience and response. *Jama*.
15. World Health Organization (2020) COVID-19 Snapshot Monitoring (COSMO) Sample questionnaire: Behavioural insights for COVID-19.
16. Likert, R. (1932). A Technique for the Measurement of Attitudes. Archives of Psychology, 140, 1–55.
17. Braun, V., & Clarke, V. (2006). Using thematic analysis in psychology. *Qual Res Psychol.* 3(2):77–101.
18. Varpio, L., Young, M., Uijtdehaage, S., & Paradis, E. (2019). Articulating the distinctions between theory, theoretical framework, and conceptual framework. *Acad Med.* Published Ahead of Print.
19. Hindmarch, W., McGhan, G., Flemons, K., & McCaughey, D. (2021). COVID-19 and Long-Term Care: the Essential Role of Family Caregivers. Canadian Geriatrics Journal, 24(3), 195.
20. Simonetti A, Pais C, Jones M, et al. Neuropsychiatric Symptoms in Elderly With Dementia During COVID-19 Pandemic: Definition, Treatment, and Future Directions. Front Psychiatry. 2020;11:579842. Published 2020 Sep 29. doi:10.3389/fpsyt.2020.579842
21. The Alzheimer Society of Ireland. (2020). Caring and Coping with Dementia During COVID-19. https://alzheimer.ie/wp-content/uploads/2020/07/ASI-Follow-Up-Covid-Report-Final.pdf
22. Campbell, P., Wright, J., Oyebode, J., Job, D., Crome, P., Bentham, P., Jones, L., & Lendon, C. (2008). Determinants of burden in those who care for someone with dementia. International journal of geriatric psychiatry, 23(10), 1078–1085. https://doi.org/10.1002/gps.2071.
23. Cooper, C., Katona, C., Orrell, M., & Livingston, G. (2006). Coping strategies and anxiety in caregivers of people with Alzheimer's disease: the LASER-AD study. Journal of affective disorders, 90(1), 15–20. https://doi.org/10.1016/j.jad.2005.08.017.
24. Gaugler, J. E., Roth, D. L., Haley, W. E., & Mittelman, M. S. (2008). Can counseling and support reduce burden and depressive symptoms in caregivers of people with Alzheimer's disease during the transition to institutionalization? Results from the New York University caregivers intervention study. Journal of the American Geriatrics Society, 56(3), 421–428. https://doi.org/10.1111/j.1532-5415.2007.01593.x.
25. Cacioppo, J. T., & Hawkley, L. C. (2009). Perceived social isolation and cognition. Trends in cognitive sciences, 13(10), 447-454.
25. Sundström, A., Adolfsson, A. N., Nordin, M., & Adolfsson, R. (2020). Loneliness increases the risk of all-cause dementia and Alzheimer's disease. The Journals of Gerontology: Series B, 75(5), 919-926.
26. Manca, R., De Marco, M., & Venneri, A. (2020). The impact of COVID 19 infection and enforced prolonged social isolation on neuropsychiatric symptoms in older adults with and without dementia: a review. Frontiers in Psychiatry, 11, 585540. 28. Flemons, K., McGhan, G., & McCaughey, D. (2022). Family Caregiving for People Living With Dementia During COVID-19: A Thematic Analysis. Journal of Family Nursing, 10748407221100553. 29. Canevelli, M., Valletta, M., Blasi, M. T., Remoli, G., Sarti, G., Nuti, F., ... & Bruno, G. (2020). Facing dementia during the COVID-19 outbreak. Journal of the American Geriatrics Society.

Bread Preservation Efficacy and Antifungal Activity of Six Wood and Leaf Essential Oils

Rhea Bhalla, Neil Bhalla, and Linda Norton

Received Sept 9 2022, revised 21 Nov 2022, accepted 21 Nov 2022, published 24 February 2023.

Abstract— Growing consumer awareness of the numerous adverse effects of chemical food preservation agents has led to an increased market interest in natural alternatives to maintain or extend the shelf life of a product. It is well established that the antimicrobial properties of essential oils allow for growth inhibition of a wide variety of pathogenic microorganisms. This study was conducted to assess the effects of tree and leaf essential oils on fungal growth and visual characteristics of bread. Packages of sliced bread were prepared with two 1.0 mL sachets of one of six essential oils and observed over a seven day period, with five samples analysed for each tested group. Measurements of fungal coverage were then taken. All six bread packages stored with essential oils presented a significantly lower level of visual indicators of spoilage compared to the control group (water). Cedar, sweet tobacco, and frankincense essential oils exhibited marked antimicrobial activity and prohibited fungal growth, extending the shelf life of bread to the end of the experimental period. The results of this study suggest that packaging bread with certain tree- and leaf-based essential oil sachets may inhibit and delay food spoilage, presenting an effective alternative to conventional synthetic preservation practices. However, significant odour produced by essential oils may indicate altered sensory properties of treated bread, limiting the applicability of this method.

I. INTRODUCTION

Due to its nearly ubiquitous consumption in various forms throughout the world, bread is recognized internationally as one of the most important dietary staples[1]. The ingredients of bread are supportive of various microorganisms[2], such as bacteria and fungi, leading to chemical, physical, and microbial spoilage. Contamination typically occurs after baking because of fungal spores that settle on exposed surfaces. The waste that accumulates as a result of this contamination causes large economic losses for both the bakery industry and the consumer[3]. Despite modern food preservation methods, food spoilage remains a significant problem. According to the Food and Agriculture Organization, food-borne moulds and their toxic metabolites lead to global losses of nearly 25% of agricultural food items yearly[4, 5].

In recent years, the numerous carcinogenic and teratogenic attributes associated with overuse of chemical antimicrobial agents in food packaging and preservation have led to increased consumer demand for manufacturers to adopt effective natural alternatives for this purpose[6]. Safe, eco-friendly, and biodegradable methods to control biodeterioration and biodegradation of food items are therefore needed to fulfill market demands for "green consumerism,"[7] while also reducing the environmental and health-related burdens placed by chemical preservatives. Growing consumer interest in effective non-synthetic food additives has reinforced the need for natural origin substances to be explored as viable means for food preservation.

Essential oils are complex mixtures of volatile oils or essences present in aromatic oils[8]. Containing a wide variety of secondary metabolites that are capable of inhibiting or slowing the growth of bacteria, yeasts and molds[9], essential oils are present as protective substances in various anatomical regions of plants and may be isolated through distillation and pressing. Various essential oils are recognized for their antifungal, antibacterial, antiviral, and antiparasitic properties[10], while also providing a broad range of health benefits. Studies investigating the antimicrobial properties of essential oils derived from floral or fruital plants, such as vanilla, lavender, and citrus peel, indicate promising potential for use in natural food preservation[2, 11]. However, current available literature indicates a significant gap pertaining to the use of tree and leaf essential oils for this purpose. Therefore, the present study was undertaken to observe the effect of tree and leaf essential oils extracted from cedar (Juniperus virginiana), sandalwood (Santalum album), sweet tobacco (Nicotiana alata), bay rum (Pimenta racemosa), teakwood (Tectona grandis), and frankincense (Boswellia thurifera) over a seven day period on microbial growth and visual characteristics of bread.

II. METHODS

Cedar, sandalwood, sweet tobacco, bay rum, teakwood, and frankincense essential oil products were procured at one hundred percent concentration with no added substances as determined by the supplier, ASAKUKI®, through the gas chromatography and mass spectrometry analysis method. Manufacture of essential oils occurred through supercritical carbon dioxide extraction from the leaves or resin of arboreal or herbaceous plants. For three days prior to usage, essential oils were stored in glass bottles in a low-humidity (35%), room temperature (24 degrees Celsius) environment away from direct sunlight. Handling and pipetting of experimental materials were completed using pipettes disinfected with a 10% solution of sodium hypochlorite. Cross contamination of essential oils was closely monitored. Further description of the six essential oils and their major chemical constituents has been provided in Table 1.

Table 1. Major chemical constituents of six tree and leaf essential oils.

Plant	Common name	Part used	Chemical compounds

Juniperus virginiana	Eastern red cedar	Resin	Cedrol, ∀-Cedrene, ∃-Cedrene, ∀-Copaene, Widdrol, Thujopsene[12]
Santalum album	Indian sandal-wood	Leaves	Palmitic acid, Oleic acid, α- and β-Santalol, Cedrol, Esters, Aldehydes, Phytosterols, Squalene[13]
Nicotiana alata	Sweet tobacco	Leaves	Phytol, solanone, Cis-5-butyl-4-methyldihydrofuran-2(3h)-one, Dihydro-β-ionone, α-Ionene, β-Damascenone, 1-Methylnaphthalene[14]
Pimenta racemosa	Bay rum	Leaves	Eugenol, β-Myrcene, α-Pinene, Linalool, Limonene[15]
Tectona grandis	Teakwood	Resin	Gallic acid, Ellagic acid, Phenolic acid, Rutin, Quercetin[16]
Boswellia thurifera	Frankin-cense	Resin	α-Pinene, α-Thujene, β-Pinene, Myrcene, Sabinene, *p*-Cymene, β-Caryophyllene, Limonene[17]

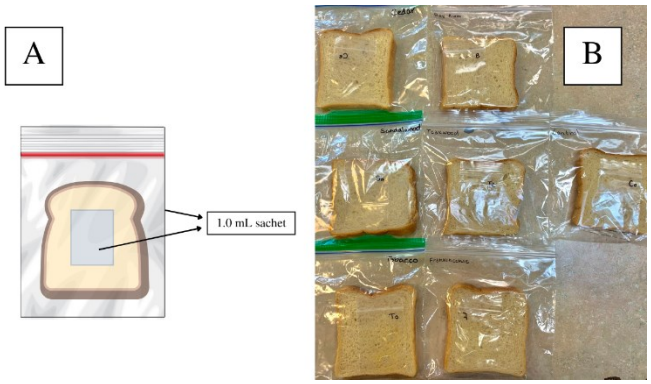


Fig. 1. (A) Depiction of packaging system with an essential oil sachet of 1.0 mL volume placed on either side of each bread slice. (B) Experimental set-up containing six tested essential oils and the control group (center rightmost package).

Sliced white bread made without the use of synthetic preservatives was obtained from a local bakery and transported to the experimental location in sealed polyethylene bags. Using a set-up similar to that of Cheng (2018)[11], each bread slice was placed in an individual Ziploc® freezer bag (17.7 × 18.8 cm, polyethylene). Resealable plastic sachets of smaller size (5.0 × 9.4 cm, polyethylene) were prepared with 1.0 mL of one of the six tested essential oils. Two packets containing the same essential oil were placed on both sides of the approximate center of each bread slice. Bread slices packaged with sachets containing 1.0 mL of distilled water served as the control group, selected to minimize the possibility of polyethylene material and liquid as confounding variables affecting bread spoilage. Bread slices were then sealed within their Ziploc® freezer bags in a low-humidity (35%), room temperature (20-22 degrees Celsius) environment away from direct sunlight for a seven day period, during which they were monitored for visual signs of fungal growth. The seven day period was selected with regard to a comprehensive review by Axel et al. (2016)[18] exploring mould biopreservation and spoilage of bread. Images of the experimental set-up are provided in Figure 1.

Following the conclusion of the experimental period, fungal growth was assessed through the use of a transparent measurement grid placed over bread samples. Data was recorded to three significant digits.

III. RESULTS AND DISCUSSION

The measured fungal coverage per each bread sample is shown in Table 2, with average growth provided in Figure 2. Microbial susceptibility to aqueous plant extracts was demonstrated in the experimental results of cedar, sweet tobacco, and frankincense essential oils, with minimal fungal growth being observed on bread slices treated with those sachets. To a lesser extent, fungicidal properties of sandalwood, bay rum, and teakwood essential oils were observed. Maximum mould growth appeared in the control group, which was treated with no essential oils. A mean fungal growth of 102 mm^2 per slice was observed the control group. Of the five experimental samples evaluated, fungal growth remains lower than the control group, suggesting that presence of two 1.0 mL essential oil sachets directly affects the fungal activity of bread. Moreover, the plant from which essential oils were extracted may determine efficacy of treatment as well as shelf life of bread. Of the tested samples, cedar, sweet tobacco, and frankincense sachets provided the greatest likelihood of a bread shelf life surpassing seven days.

Data obtained from the present study are in close concurrence with research on the antimicrobial properties of essential oils. Therefore, the inhibitory effects observed may be attributed to volatile compounds released from the essential oil sachets that inhibited mould growth. The antifungal activities of the essential oils may be attributed to their major components, which act as active inhibitors of bacteria, yeasts and filamentous moulds[19], thereby preventing pathogenic invasion of the plants from which they are derived. The specific antimicrobial constituents of the six essential oils tested in this study are as follows: (1) cedar, containing cedrol and thujopsene[20], (2) sandalwood, containing α-santalol[21], (3) sweet tobacco, containing defensin (NaD1 and NaD2)[22], (4) bay rum, containing eugenol[15], (5) teakwood, containing ethanolic, methanolic, ethyl acetate extracts[23], and (6) frankincense, containing monoterpenes[24]. Results of this study indicate that diffusion of these volatile antimicrobial compounds through a polyethylene layer creates an antifungal effect on sliced bread.

Table 2. Coverage of fungal growth per each of five tested bread slices (mm^2), provided to three significant digits.

Treatment	Fungal Growth Per Bread Slice (mm^2)				
	1	2	3	4	5
Cedar	0.00	3.18	4.00	0.00	1.59
Sandalwood	5.56	17.5	4.76	34.9	9.52
Sweet tobacco	7.94	2.38	0.00	2.38	3.20
Bay rum	88.9	66.7	0.00	73.0	23.8
Teakwood	170	70.0	30.2	96.8	19.0
Frankincense	0.00	0.00	7.94	15.1	4.76
Control	181	127	25.4	41.4	135

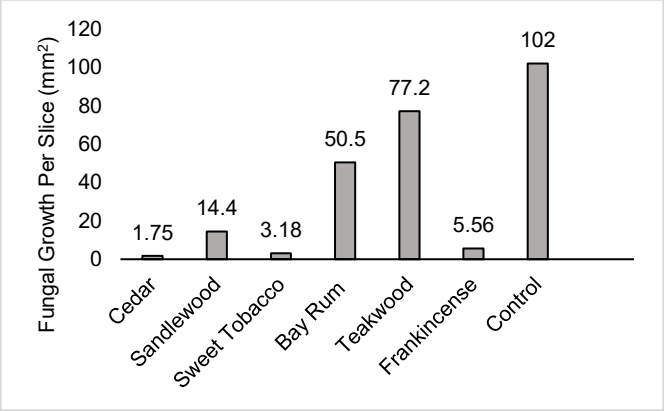


Fig. 2. Graphical comparison of mean fungal growth per tested group (mm^2). Numerical values are provided above each bar.

It should be noted that the significant odour produced by the essential oils used in this study may limit the applicability of this method to treat bakery products that are consumed without further alterations. Further research is needed to evaluate the sensory properties of bread treated with tree and leaf essential oils as a method of preservation. Special emphasis may be placed on intense residual flavours and aromas, as well as characteristics such as symmetry of form, quality of crust, colour of crumb, colour of crust, taste, texture, aroma, and grain of bread. The main advantage of the preservation method described in the present study is in the demonstrated ability of tree and leaf essential oils to extend the shelf life of bread in the absence of synthetic preservatives that may pose a concern to human health and act as a contaminant of the environment. Bread slices treated with one of six essential oils or a control sample are shown at the conclusion of the experimental period in Figure 3.

Fig. 3. Bread slices after seven days at room temperature. Different treatments were carried out: (A) cedar, (B) sandalwood, (C) sweet tobacco, (D) bay rum, (E) teakwood, (F) frankincense, (G) distilled water (control). Opposite sides of the same bread slices are presented in the below image.

It may also be considered that although essential oils are commonly used as food additives, they may cause allergic reactions and adverse health effects in certain cases[25]. Further research may be required to ensure the safety of this preservation method.

The use of essential oils was previously applied to reduce the growth of pathogenic microorganisms in bread. Similar to the present study, earlier research focused on improving bread shelf life while inhibiting and delaying fungal growth. However, these studies were largely focused on floral and fruital plant extracts and did not examine tree- and leaf-based essential oils. Salim-ur-Rehman et al. (2007)[2] studied the effects of malta (Citrus sinensis) and mossumbi (Citrus sinensis) citrus peel essential oils on the microbial growth and sensory characteristics of bread. The results showed that maximum inhibitory effect was achieved against molds and bacteria by spraying malta peel essential oil on bread. However, the direct application of the essential oil significantly affected sensory characteristics of bread, such as taste and aroma.

In a similar study, Cheng (2018)[11] explored various essential oils not evaluated in the present study for their antifungal activity against moulds isolated from contaminated bread, with special emphasis on cinnamon essential oil. Active packaging combined with 500 µL to 1000 µL of cinnamon essential oil in sachets increased the shelf life of bread slices packaged in plastic bags by over 14 days. In accordance with the present study, polyethylene sachets containing essential oils were found to be an effective means for the preservation

of sliced bread. Moreover, an overview of the varying effects of essential oils on different bread types and compositions was provided, with white breads such as the ones used in this study experiencing fungal inhibition for extended periods of time. Passarinho et al. (2014)[26] developed antimicrobial sachets containing different concentrations of oregano essential oil and evaluated their antimicrobial efficiency on agar medium and on sliced bread. They found that sachets containing oregano oil showed in vitro antimicrobial effects against common microorganisms and reduced the growth rates of yeasts and moulds on sliced bread.

Despite the breadth of research on the bread preservation efficacy of certain essential oils, none of these reports, at the current time of writing, provide an in-depth analysis of the antimicrobial properties of tree- and leaf-based essential oils in the context of the present study. Therefore, the data provided in this research supporting the use of cedar, sweet tobacco, and frankincense essential oil sachets to lengthen the shelf life of bread may assist in the development of a novel approach. Not only does this method satisfy market demands for natural modes of food preservation, it reduces economic losses related to premature spoilage of bakery products. Considering both the results of this study as well as past research pertaining to this field, tree- and leaf-based aqueous essential oil extracts provide an effective antimicrobial alternative to synthetic chemical preservatives for bread.

IV. CONCLUSION

In the present study, six tree and leaf essential oils were proposed as natural antifungal agents. The evaluation of aqueous extracts placed in close contact with sliced bread over a seven day period confirmed the food preservation efficacy of these substances. The greatest antimicrobial activity was observed in bread slices treated with cedar, sweet tobacco, and frankincense essential oils. Sandalwood, bay rum, and teakwood essential oils inhibited fungal growth to a lesser extent. The application of tree and leaf essential oils in 1.0 mL polyethylene sachets resulted in an effective strategy to reduce fungal contamination while extending shelf life. For this reason, cedar, sweet tobacco, and frankincense essential oils may be a good candidate to replace traditional synthetic preservatives, thereby satisfying consumer demands and trends toward natural food preservation methods. Further research may wish to explore the application of a greater variety of tree and leaf essential oils while also considering their effects over a longer period of time, as well as impacts of polyethylene sachets of varying permeabilities. Sensory effects of these products as natural food preservation agents, such as alterations in taste, aroma, and texture of bread, may also be examined.

ACKNOWLEDGMENT

This study was conducted using the facilities of Notre Dame High School. The authors acknowledge the contributions of Jessie Estrada, Valeria Duran, Christal Robles, and Frances Otoide.

REFERENCES

1. Dewettinck, K., Van Bockstaele, F., Kühne, B., Van de Walle, D., Courtens, T. M., & Gellynck, X. (2008). Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science*, *48*(2), 243-257.
2. Rehman, S., Hussain, S., Nawaz, H., Ahmad, M., Murtaza, M., & Rizvi, A. (2007). Inhibitory Effect of Citrus Peel Essential Oils on the Microbial Growth of Bread. Pakistan Journal of Nutrition 6 (6): 558-561.
3. Axel, C., Zannini, E., & Arendt, E.K. (2017).Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. Crit. Rev. Food Sci. Nutr, 57, 3528–3542.
4. Pittet, A. (1998). Natural occurrence of mycotoxins in foods and feeds-An update review. Revue de Medecine Veterinaire. 149:479-492.
5. Singh, P., Shukla, R., Kumar, A., Prakash, B., Singh, S., & Dubey, N.K. (2010). Effect of Citrus reticulata and Cymbopogon citratus essential oils on Aspergillus flavus growth and aflatoxin production on Asparagus racemosus. Mycopathologia; 170:195-202.
6. Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., & Coton, E. (2016). In vitro and in situ screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. Food Control 2016, 60, 247–255.
7. Smid, E.J., & Gorris, L.G.M. (1999). Rehman MS. (Ed.), Handbook of food preservation. New York: Marcel Dekker, 285-308.
8. Leyva-López, N., Gutiérrez-Grijalva, E. P., Vazquez-Olivo, G., & Heredia, J. B. (2017). Essential Oils of Oregano: Biological Activity beyond Their Antimicrobial Properties. *Molecules (Basel, Switzerland)*, *22*(6), 989. https://doi.org/10.3390/molecules22060989
9. Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., & De Feo, V. (2013). Effect of Essential Oils on Pathogenic Bacteria. *Pharmaceuticals*, *6*(12), 1451–1474. MDPI AG. Retrieved from http://dx.doi.org/10.3390/ph6121451
10. Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *Journal of applied microbiology*, *86*(6), 985-990.
11. Cheng, M. (2018). Antimicrobial Activity of Essential Oils and Their Application in Active Packaging to Inhibit the Growth of Molds on Bread. Department of Food Science and Agricultural Chemistry McGill University, Montréal, Québec.
12. Lawrence, B.M. (1993). Cedarwood oil. Essential Oils (1988-1991). Carol Stream, IL, Allured Publishing Corp., pp 176-177
13. Zhang, X. H., da Silva, J. A., Jia, Y. X., Zhao, J. T., & Ma, G. H. (2012). Chemical composition of volatile oils from the pericarps of Indian sandalwood (Santalum album) by different extraction methods. *Natural product communications*, *7*(1), 93–96.
14. Popova, V., Ivanova, T., Nikolova, V., Stoyanova, A., Docheva, M., Hristeva, T., Damyanova, S., & Nikolov, N. (2017). Chemical composition of Nicotiana alata. *Journal of Pharmaceutical Sciences and Research, Vol*. 9(11), 2017, 2045-205.
15. El-gizawy, H.A., Mosaad, Y.O., Gobba, N.A.E.K., & Hussein, M.A. (2018). Chemical Composition of the Essential oil of The Leaves of Pimenta diocia (L.) Merr. & Pimenta racemosa (Mill.) cultivated in Egypt and Evaluation of Their in-vitro Antioxidant and Antidiabetic Activities. *International Journal of Phytomedicine.* 10(4):226-234.
16. Naira, N. (2010). Isolation of phenolic compounds from the methanolic extract of Tectona grandis. *Research Journal of Pharmaceutical Biological and Chemical Sciences*, *1*(2), 221-225.
17. DeCarlo, A., Johnson, S., Ouédraogo, A., Dosoky, N.S., & Setzer, W.N. (2019). Chemical Composition of the Oleogum Resin Essential Oils of Boswellia dalzielii from Burkina Faso.Plants 2019, 8, 223.
18. Axel, C., Zannini, E., & Arendt, E.K. (2016). Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. Critical Reviews in Food Science and Nutrition. 57(16):00-00.
19. Lee, H. C., Cheng, S. S., & Chang, S. T. (2005). Antifungal property of the essential oils and their constituents from Cinnamomum osmophloeum leaf against tree pathogenic fungi. Journal of the Science of Food and Agriculture, 85(12), 2047-2053. doi:10.1002/jsfa.2216.
20. Mun, Sung Phil & Prewitt, Lynn. (2011). Antifungal Activity of Organic Extracts from Juniperus virginiana Heartwood against Wood Decay Fungi. Forest Products Journal. 61. 443-449. 10.13073/0015-7473-61.6.443.
21. Santha, S., & Dwivedi, C. (2015). Anticancer Research, June 2015, 35 (6) 3137-3145.
22. Afroz, M., Akter, S., Ahmed, A., Rouf, R., Shilpi, J.A., Tiralongo, E., Sarker, S.D., Göransson, U., & Uddin, S.J. (2020). Ethnobotany and Antimicrobial Peptides From Plants of the Solanaceae Family: An Update and Future Prospects. *Front. Pharmacol.* 11:565. doi: 10.3389/fphar.2020.00565

23. Lanka, S. (2017). ANTIMICROBIAL ACTIVITIES OF TECTONA GRANDIS LEAF AND BARK EXTRACTS. EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH. 4. 245-248.
24. Di Stefano, V., Schillaci, D., Cusimano, M. G., Rishan, M., & Rashan, L. (2020). In Vitro Antimicrobial Activity of Frankincense Oils from *Boswellia sacra* Grown in Different Locations of the Dhofar Region (Oman). *Antibiotics (Basel, Switzerland)*, *9*(4), 195. https://doi.org/10.3390/antibiotics9040195
25. Sharma, S., Barkauskite, S., Jaiswal, A., & Jaiswal, S. (2021). Essential oils as additives in active food packaging. https://doi.org/10.1016/j.foodchem.2020.128403
26. Passarinho, A.T.P., Dias, N.F., Camilloto, G.P., Cruz, R.S., Otoni, C.G., Moraes, A.R.F. and Soares, N.d.F.F. (2014). Bread Preservation through Antimicrobial Sachet. *J Food Process Eng*, 37: 53-62. https://doi.org/10.1111/jfpe.12059